Chemical Toxicity of the Granulocyte

by John C. Marsh*

The effect of chemicals, including pharmacologic agents, on blood granulocytes, may be considered in terms of the effects on the function of mature cells as well as on their number. In turn, the number of cells can be influenced by chemicals which affect production, destruction or distribution within the blood.

Neutrophil functions which can be inhibited by chemical agents include chemotaxis, phagocytosis, degranulation, the metabolic burst related to membrane perturbation and intracellular killing. Some drugs can influence multiple neutrophil functions.

Neutrophil production may be inhibited by toxic chemicals with a predictable effect, such as cancer chemotherapeutic drugs, where the effects are dose related, or the process may be influenced in an unpredictable (idiosyncratic) fashion, occurring only in a small proportion of patients exposed. Ineffective granulopoiesis with intramedullary death may be seen as a result of exposure to some drugs, as may increased neutrophil destruction, sometimes via an immunologic mechanism.

Predictable neutrophil production inhibition by anticancer agents depends on dose, schedule, route of administration, metabolic integrity of organ systems such as liver and kidney, and the proliferative state of the marrow. For a given drug, factors which determine neutrophil toxicity include its mechanism of action, cytotoxic concentration, pharmacokinetics, metabolic and excretory pathway and target cells in the marrow. The use of assays for clonogenic hematopoietic precursor cells is allowing correlation with more traditional toxicologic methods and may be helpful in predicting specific hematologic toxicity in man, prior to actual clinical trials. The agar diffusion chamber assay for neutrophil-macrophage committed colony-forming cells is particularly useful since it allows in vivo exposure of the target cells to the agents being studied.

Consideration of the influence of chemical agents on circulating blood granulocytes may be divided into effects on the function of the mature cell and on the number of circulating cells in the blood. In turn, cell number may be considered from the standpoint of decreased production by the marrow, increased destruction, or changes in the ratio of circulating cells, which are counted, and marginated cells, which are not.

In this discussion, consideration will be limited to neutrophilic granulocytes, not only because they are more numerous than eosinophilic and basophilic granulocytes, but because much more information is available about their function, regulation of production and kinetics.

Neutrophils normally leave the bone marrow as mature cells, with a complement of both primary (azurophilic) and secondary (specific neutrophilic) granules, which are full of various enzymes which perform specific tasks. Mature cells are recognized as such in the blood as either mature polymorphonuclear (PMN) cells or as slightly less mature band forms. They enter the blood and remain in it with a half-life of about 7 hr and leave it randomly, passing between capillary endothelial cells into the tissues where they perform their major task, that of being the advance guard of phagocytes against invading microorganisms. Clearly, anything that interferes with either the normal function of these cells or their number can predispose the host to infection, which may be lethal.

Neutrophil Function

In considering neutrophil function (1), it is important to distinguish between studies in vitro, in

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which a concentration to isolated normal cells whose function is then measured, and *in vivo* stuides, in which PMN's are removed from animals or patients who have been given the drug under study, and whose function is then compared to that of normal cells. The latter type of study is more meaningful but, unfortunately, information of this type is not always available.

What does a mature PMN do? Among those functions which can be measured are adherence, chemotaxis, phagocytosis, degranulation, and a metabolic burst accompanied by intracellular killing of ingested microorganisms.

By adherence is meant the attachment of cells to vascular endothelium prior to their leaving the blood by passage between these same endothelial cells. The complex changes occurring in diabetes, as well as in the presence of ethanol, have been reported to inhibit this process. Epinephrine can cause a very rapid increase in the concentration of PMN's in the circulating blood by demargination, presumably also by interfering with PMN adherence. Histamine, iron oxide, and dextran on the other hand, appear to enhance the process of margination, causing a "pseudoneutropenia."

Chemotaxis means the directed movement of cells in response to a concentration gradient of a mediator. Among the known chemotactic factors are bacterial components, those derived from the complement system, those derived from Hageman factor activation and those derived from the PMN itself, including cyclic AMP (cAMP) and certain prostaglandins. Endotoxin, cellular necrosis, antigen-antibody complexes, and nonspecific tissue damage may attract neutrophils by various combinations of these mechanisms.

The process of chemotaxis is most commonly studied *in vitro*, using a chamber in which the cells to be studied are separated from the chemotactic stimulus by a filter which can then be examined for the extent of PMN migration through it (2). Its *in vivo* counterpart is the skin window (3) in which coverslips are placed over a skin abrasion, with or without a bacterial stimulus, and examined quantitatively over time for the rate of appearance of PMN's.

Microtubules within the cell are important in the chemotactic process. The function of these structures is inhibited by colchicine, vincristine, and vinblastine, as well as halothane. Colchicine, as well as actinomycin D, has also been reported to interfere with the elaboration of a chemotactic factor produced by PMN's after they ingest urate crystals.

The PMN's of colchicine-treated patients, however, tic responses *in vitro*, but a decreased response to inflammatory skin windows. Increased levels of cAMP inhibit chemotaxis, and the action of histamine, various β-adrenergic agents, theophylline, and certain prostaglandins has been explained in this way. On the other hand, increased levels of cyclic GMP (cGMP) enhance chemotaxis, and various cholinergic drugs, as well as levamisole, are thought to act in this fashion. High doses of vitamin C also enhance chemotaxis.

Several antiinflammatory agents are *in vitro* inhibitors of chemotaxis. These include hydrocortisone, methyl prednisolone, chloroquine, quinine, and phenylbutazone, while aspirin is without effect. The relatively high concentrations that were needed to show this effect, however, raise some question about the significance of the observation. The intravenous injection of a large dose of hydrocortisone, however, did inhibit the accumulation of PMN's at skin windows as well as cause an increase in the rate of marrow input of PMN's into the blood (4).

Other inhibitors of chemotaxis, based on *in vitro* studies, include the antibiotics, tetracycline, rifampin, chloramphenicol, and amphotericin B. The tranquilizer chlorpromazine, penicillamine, ethanol, heparin, and caffeine have also been identified as inhibitors.

Phagocytosis is the engulfment of a particle and its sequestration in an intracellular vacuole. One can measure both the rate of uptake of particles over a period of time, or the number of particles per cell at a specific time. The percentage of cells which contain ingested particles can also be measured. The phagocytic process is also inhibited by cAMP and enhanced by increased intracellular levels of cGMP, and therefore is influenced by the same agents which affect these cyclic nucleotides, mentioned above under chemotaxis. Elevated levels of the sugars galactose and glucose inhibit phagocytosis, thus providing a suggestive explanation for the increased susceptibility to infection seen in galactosemia as well as the much more common diabetes mellitus. Colchicine has been reported both to inhibit and not to influence phagocytosis.

Degranulation is the delivery of the granule contents of the PMN into the phagocytic vacuole or into the extracellular environment. Intracellular degranulation is inhibited by colchicine and vinblastine through their effect on microtubules, and by theophylline, histamine, and β-adrenergic agents. The process is enhanced by cholinergic agents. Degranulation into the extracellular environment is inhibited by corticosteroids and chloroquine.

Phagocytosis or, indeed, even perturbation of the

oxygen consumption, increased glucose utilization, including activation of the hexose monophosphate shunt, generation of a superoxide anion, which is important in intracellular killing, and chemiluminescence. Colchicine and vinblastine have been reported to interfere with the generation of superoxide anion.

The antimicrobial systems in the PMN are those which are oxygen dependent, including that mediated by myeloperoxidase, inhibited by various sulfonamides and antithyroid agents, and oxygen independent systems, which include killing mediated by lysozyme, lactoferrin and various cationic proteins found in the granules.

Lithium carbonate has well defined stimulatory effects on leukocyte production and is being used to protect against the neutropenia produced by various antineoplastic agents. When the PMN's of normal volunteers were studied before and after the ingestion of lithium, they were found to have normal mobilization to skin windows, nitroblue tetrazolium reduction (as a measure of superoxide anion production), normal chemotaxis in a Boyden chamber to bacterial products, normal phagocytosis, and chemiluminescence but impaired intracellular killing of ingested *Staph. aureus* (5). The importance of this observation relative to the beneficial effect of lithium on neutrophil production remains to be determined.

Ethanol has multiple inhibitory effects on the PMN: not only is there impaired production, but there is diminished cellular adherence, decreased chemotaxis *in vitro* (although skin window tests of acutely intoxicated patients are normal) and diminished phagocytosis.

Diabetes has been associated with decreased PMN adherence, chemotaxis, phagocytosis and intracellular killing.

Among the defects described in chronic renal failure and the resulting symptom complex known as uremia is impaired chemotaxis, but phagocytosis and intracellular killing of organisms have been found to be normal in most studies.

Reduced Neutrophil Numbers (Neutropenia)

In theory, a reduction in the number of circulating granulocytes in the blood (6) may come about by inadequate production in the marrow, excessive peripheral destruction or utilization, or by a shift in the circulating granulocytes into the blood marginal pool, a situation which has been referred to as "pseudoneutropenia".

the most common effect of pharmacologic agents. Reduced granulocytopoiesis may be predictable or unpredictable (idiosyncratic).

Predictable neutropenia is the type seen commonly with cancer chemotherapeutic agents, which do not distinguish between normal and cancer cells. It develops slowly and is dose-dependent. It may arise from direct stem cell damage, from interruption of DNA synthesis, the binding or depolymerization of DNA, inhibition of mitosis, or interference with protein synthesis. Examples, in addition to most antineoplastic agents, are chloramphenicol, rifampin, ristocetin, benzene, ethanol, and nitrous oxide. We will return to a more detailed consideration of this type of toxicity, particularly with respect to stem cells, in the discussion below.

Idiosyncratic drug-induced interference with granulopoiesis may be of two types: that which is slow to develop and dose-dependent and that which appears to be a hypersensitivity reaction and occurs rapidly. In the former type, certain types of people are at increased risk: the old more than the young, female more than male, and white more than black. It is thought to be an individual sensitivity of DNA synthesis, at least with the prototype which is phenothiazine intoxication. The onset is usually between two and three weeks after drug ingestion, and the incidence is of the order of 1 in 1000-2000. Among the drugs implicated in this type of delayed idiosyncratic inhibition of granulocyte production are phenothiazines, chloramphenicol, acetaminophen (Tylenol), carbenicillin, butazolidine, propylthiouracil, sulfa drugs, and procaine amide.

The rare idiosyncratic neutropenia that appears to be a hypersensitivity reaction develops within a few days of beginning drug ingestion. It is usually associated with prior ingestion of the drug and with eosinophilia. Recovery is usually prompt, but may be slow. Drugs causing it often contain a benzene ring; they include chloramphenicol, gold salts, phenylbutazone, sulfas, INH, indomethacin, procaine amide, ampicillin, nitrofurantoin, propylthiouracil, and chlorothiazide.

Several drugs are associated with what seems to be ineffective granulopoiesis, that is, an intramedullary death of granulocyte precursors. This is the picture seen with vitamin B_{12} or folate deficiency, and has been described with diphenylhydantoin, pyrimethamine, ethanol, and chloramphenicol. This type of neutropenia develops slowly, and is often dose-dependent and predictable.

Turning to increased granulocyte destruction, the neutrophil analog of hemolytic anemia, some drugs cause an immune destruction of granulocytes, mediated through an antibody. This may

viously exposed to the drug, and within a few days after initial exposure. The classic drug so implicated is aminopyrine; serum from a sensitized patient and the drug together will produce neutropenia in a normal patient, but neither is sufficient when given alone. Rarely, phenylbutazone and sulfa drugs produce neutropenia through a similar mechanism. Antibodies which agglutinate neutrophils *in vitro* have been found in some patients with neutropenia and with a history of ingesting the following drugs: gold salts, chloroquine, barbiturates, methyldopa, and phenothiazines. The exact significance of these agglutinins in producing the neutropenia is uncertain.

Redistribution neutropenia or "pseudoneutropenia" is an increase in the marginal granulocyte pool at the expense of the circulating pool, but with no net change in the total blood granulocyte pool. Histamine, dextran and iron oxide have all been reported to effect this redistribution.

Neutropenia Due to Cancer Chemotherapeutic Agents

As discussed above, neutropenia due to treatment with most antitumor drugs (7) is due to interference with the production of bone marrow neutrophils. This may be manifest at several points in the granulocyte production pathway, but with the advent of stem cell assays, the effects of many agents on various blood cell precursors has received close attention (8).

The Achilles heel of cancer chemotherapy is bone marrow depression, particularly neutropenia with its attendant risk of infection. Nearly all of the agents currently in routine clinical use and under investigation are capable of producing this dangerous side effect. Vincristine, bleomycin, L-asparaginase, and estramustine are among those in which neutropenia is generally mild or absent, and of little clinical importance.

The potential for the production of neutropenia by a specific drug is a function of the dose, the route of administration, the schedule, the metabolic integrity of the host, and the proliferative state of the bone marrow, and its various components.

The depth of neutropenia and its duration can be shown to be directly related to the dose of cyclophosphamide in various groups of patients with solid tumors. Patients with a limited bone marrow reserve due to prior destruction by irradiation or infiltration by tumor are often more sensitive.

The influence of route of administration can be seen by the fact that doses of 5-fluorouracil or adriamycin can be given directly into the liver,

would cause otherwise prohibitive toxicity.

An example of the importance of schedule is shown by the experience with a constant dose of cytosine arabinoside. When the time of a continuous infusion was lengthened from 24 to 48 to 96 hr, the degree and duration of neutropenia was progressively prolonged. This is the result of a larger number of neutrophil precursors in the marrow coming into contact with this DNA synthesis phase-specific agent in the longer time period.

The importance of the metabolic integrity of a specific organ is shown by the fact that adriamycin, commonly used in lymphomas and in breast, bladder, and testicular cancer, may cause lethal toxicity in a patient with hepatic decompensation in a dose that would otherwise be well tolerated. In such a patient, the dose must be decreased by 50 to 75% because the metabolic degradation of the active cytotoxic agent is impaired. A similar caution exists for the use of methotrexate in the patient with impaired renal function.

The importance of considering the proliferative state of the bone marrow is shown by the fact that a specific dose of cyclophosphamide is well tolerated initially without significant leukopenia, but a second identical dose given 12 days later produces a sharp fall in the level of the circulating blood neutrophils. This observation can be explained by the increased sensitivity of the neutrophil precursors, which are rapidly proliferating to compensate for the cytotoxicity and cell kill caused by the first dose.

Until recently, our knowledge of bone marrow toxicity in man resulting from anticancer agents has been empirically derived from clinical trials over the last 35 years.

Table 1 contrasts the nadir and recovery time of blood neutrophils following radiation therapy as might be given for Stage II Hodgkin's disease, an alkylating agent such as nitrogen mustard, an antimetabolite and a nitrosourea. It is clear from the differences that there are marked variations as to which precursor cells in the neutrophil series are primarily affected. The very late toxicity after the nitrosoureas suggests that they kill an early precursor cell, while alkylating agents affect more differentiated precursors. As an oversimplification, it may be stated that phase-specific agents such as hydroxyurea or cytosine arabinoside kill only cells which are in DNA synthesis, while alkylating agents can have multiple targets, including cells which are in different phases of the cell cycle. Nitrosoureas can, in addition, affect very slowly or nonproliferating multipotent hematopoietic stem cells.

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Table 1.

four anticancer agents.

	Nadir (days)	Recovery (days)
X-ray "mantle"	14	90
Alkylating agent (nitrogen mustard)	10	21
Antimetabolite (cytosine arabinoside)	14	28
Nitrosourea (CCNU)	35–42	50–60

What clues are available from clinical observations to help the physician decide whether a specific dose of chemotherapy can be given safely? The integrity of the normal bone marrow can be presumed to be compromised if neutropenia develops earlier than a week from a myelotoxic agent, since the normal marrow granulocyte storage pool should be able to maintain a normal circulating neutrophil level for that time, even if the production of mitotically active cells is completely halted. The total white blood cell count, if normal, can be misleading, since the differential count may be predominately lymphocytes. The absolute number of blood neutrophils should be determined. Tests of bone marrow granulocyte reserve can be used: these include the neutrophil increment in response to endotoxin, etiocholanolone or prednisone. These tests are relatively crude and can indicate the presence or absence of a granulocyte storage pool in the marrow but not whether it is reduced in size.

If there is a significant increase in the ratio of band to segmented cells in the blood, this indicates that the marrow granulocyte reserve is inadequate and that the marrow is still releasing relatively young cells into the circulation to maintain a normal blood granulocyte pool.

What factors do we need to know to understand the potential for bone marrow toxicity of a specific myelosuppressive drug? They include its biochemical mechanism of action, the effective concentration required for cell killing, its pharmacokinetics as determined by volume of distribution, metabolism and excretion, and the target stem cells in the bone marrow which are sensitive to its action.

Effects of Antitumor Agents on Bone Marrow Stem Cells

As discussed in an earlier paper in this symposium, various assays for hematopoietic stem cells are available (8). The oldest and perhaps most significant is that for the multipotent, uncommitted cell, mature blood cell in the spleen of the irradiated mouse, the CFU-S (9). This cell normally proliferates rather slowly (only a small proportion is killed by exposure to tritiated thymidine), but the rate of proliferation speeds up if the compartment is reduced in size. It meets the definition of a stem cell in that it is capable of both self-renewal and differentiation. A severely damaged population of marrow stem cells proliferates more rapidly than a less severely damaged one, and there is evidence (7) that with severe damage (e.g., less than 10% survival of the original compartment) self-replication takes priority over differentiation. The CFU-S compartment seems to be subject to competing demands for differentiation; i.e., an anemic animal may be less able to recover normal granulocyte production than a normal one, because of the conflicting need for red cell as well as white cell production.

Since an assay for the uncommitted stem cell does not exist in man, attention has turned to *in vitro* precursor cell assays, which measure clonogenic cells committed to specific lines of blood cell formation. The erythrocyte-committed cells, CFU-E and BFU-E, are dependent on erythropoietin. The CFU-C assay (10) measures those cells which can give rise to granulocytes and monocyte-macrophages in soft agar or methycellulose. Their growth depends on colony-stimulating activity, at least *in vitro*, and they are subject to various regulatory factors which are just becoming clarified.

The granulocytosis produced by lithium has been reported to be due to an increased release of colony-stimulating factor (11) and also to an increased proliferation of pluripotent stem cells (CFU-S) (12).

Our laboratory for the last several years has been using a modification of the CFU-C assay, which utilizes an agar diffusion chamber (ADC) and measures an agar diffusion chamber precursor cell (ADCPC) (13). It forms colonies composed of granulocytes and macrophages and has two major advantages over the CFU-C assay: it does not require a source of colony-stimulating activity, and the drug to be tested can be injected into the chamber-bearing mouse and thus undergo *in vivo* transformation as it interacts with the bone marrow clonogenic cells.

We have done two types of assay with this technique: dose-response experiments in which the bone marrow is exposed to the drug *in situ* (only in the mouse) and then assayed for surviving colony forming cells); and dose-response experiments in which the mouse, human, or canine marrow is exposed to the drug while it is suspended in the

ADC.

(IV), and after a fixed time (usually 18 hr), the chamber is transplanted into a normal mouse for incubation for 7-14 days, depending on the species. In this way, the sensitivity of mouse marrow in the chamber can be compared with that *in situ*, and also with marrow from dog and man. Correlations with toxicology studies in animals and Phase I studies in man can be made. It should be possible then, to predict the potential for the production of neutropenia by a new drug in humans without ever exposing patients to it. Curves relating stem-cell survival to drug dose are of two major types, although sometimes complex curves are seen.

The first type is an exponential curve, reflecting first-order kinetics of clonogenic cell kill. This means that the population of colony- forming cells in the marrow is killed in a fixed proportion for each increment of a drug over the range studied, and that the dose-survival curve is a straight line when plotted on semi-logarithmic paper. Doses which produce a specific cell survival (D_{37} = that dose required to kill 63% of the cells) can be calculated from the exponential portion of the curve and these doses compared for different species. When we studied adriamycin (Fig. 1), we found similar sensitivities for mouse marrow *in situ*, in the chamber, and human marrow (D_{37} values of 8, 8, and 9 mg/kg) but dog marrow was much more sensitive (3.5 mg/kg)(*14*). The dog is known from toxicologic studies to be the most sensitive of these species to adriamycin, whose limiting toxicity is granulocytopenia. The adriamycin derivative, AD-32, has a similar order of marrow colony-forming cell sensitivity, although human cells are relatively more sensitive than to adriamycin. It was predicted that granulocytopenia would be the limiting clinical toxicity for AD-32 also; such, indeed, is the case (*15*). Carminomycin, a Russian anthracycline derivative, has an exponential dose-survival curve, while the anthracycline marcellomycin, which has no leukocyte toxicity in mice, has virtually a flat dose-survival curve.

Other drugs with exponential dose-survival curves for ADCPC include vinblastine, cyclophosphamide, 5-fluorouracil, *cis*-platinum, and pyrazofurin. There are, however, definite differences in the slopes of the curves for different species, and the species sensitivities vary from drug to drug; they are not predictable (*16*).

The other major type of dose-survival curve is the plateau, in which only a limited proportion of colony-forming cells is killed, regardless of the dose of drug given (Fig. 2). This is due to the drug killing cells only in a certain phase of the cell cycle, and, in some instances, the ability of the drug to cause
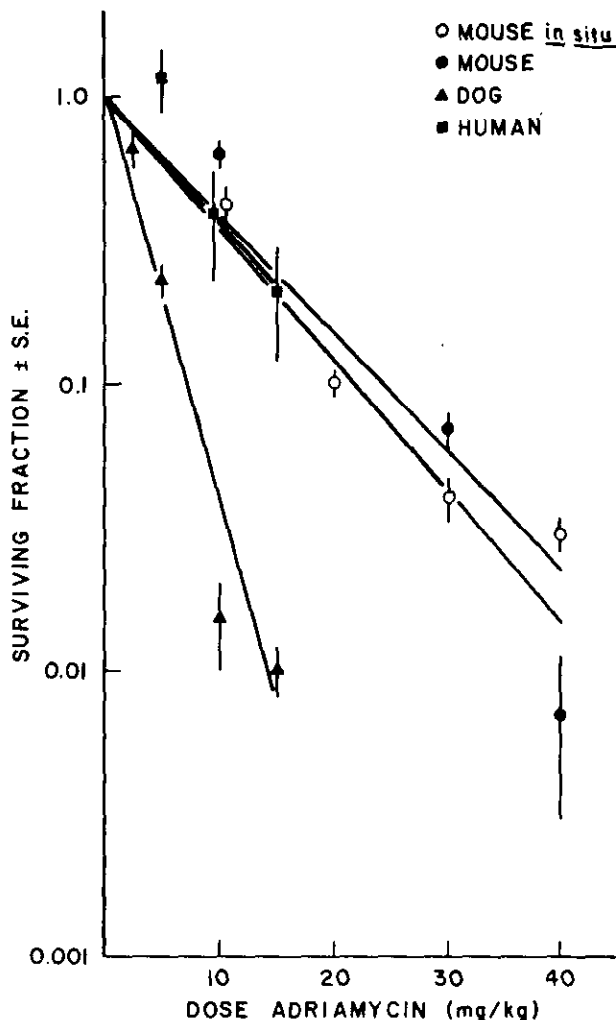

FIGURE 1. Dose-survival curves of bone marrow agar diffusion chamber precursor cells from different species 18 hr after adriamycin.

delay or block in passing through the cycle. The location of the plateau is determined also by the pharmacokinetics of the drug, i.e., how long the population is exposed to a cytotoxic concentration of drug. Since this is the same when the marrows from different species are exposed in the chamber, a true picture of comparative species sensitivities can be obtained. Drugs which produce plateau dose-survival curves include the antifolates methotrexate, triazinate (Baker's antifol), a trimethoxyquinazoline derivative about to enter clinical trial, cytosine arabinoside (*17*), and 5-azacytidine. Estramustine produces a plateau for dog and human ADCPC, but an exponential curve for mouse marrow cells. Differences in species sensitivity are
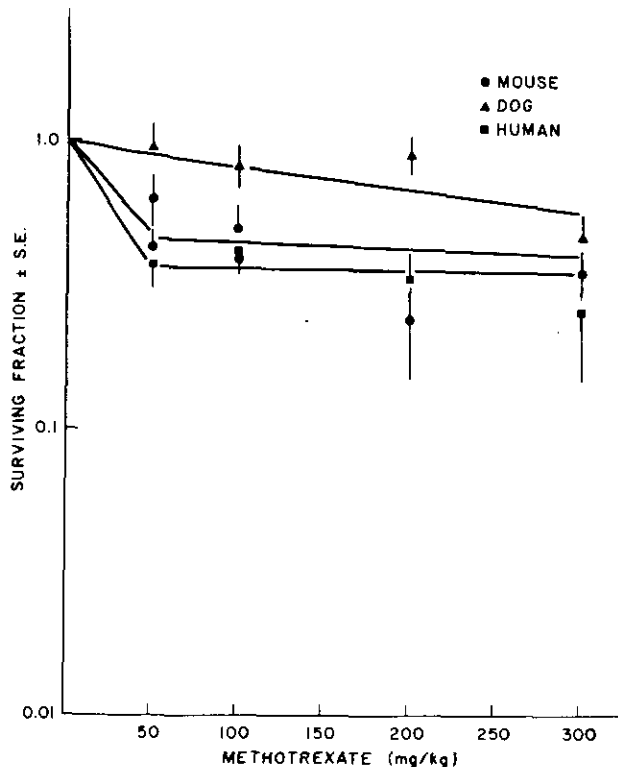
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FIGURE 2. Dose-survival curves of bone marrow agar diffusion chamber precursor cells from different species 18 hr after methotrexate.

usually slight, but again, are not consistent or predictable.

The effect of two-drug combinations can also be tested in this system. A combination of methotrexate and 5-fluorouracil is of current clinical interest and appears to be synergistic in killing some kinds of tumors. Naturally, the therapeutic index depends on whether or not this combination also kills marrow colony-forming cells synergistically as well. Some preliminary data with marrow from all three species suggests that it does not. Likewise, it has been shown that pretreatment of mice with allopurinol will protect mice from 5-FU lethality (18). We have observed that allopurinol protects marrow ADCPC in mice from this drug, which may explain at least part of the toxicologic effect (19).

In conclusion, the leukocyte-committed colony forming cell (ADCPC or CFU-C) is probably the most relevant population for the study of hematological toxicity in man, since leukopenia is the most potentially lethal complication of such toxicity. It can be studied serially over a period of time in the blood or marrow, as well as evaluated for sensitivity to specific drugs in specific individuals. The assay is potentially of value in predicting the neutropenia in man without the necessity for clinical trial. In combination with assays of tumor stem cell drug sensitivity, it may be possible to design optimal chemotherapy for cancer patients in a much more rational fashion than hitherto has been the case.

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